unknown. Understanding this transition is crucial as potentiating molecules like anesthetics and alcohols are believed to affect transition barriers or the relative free energy of states [1]. To investigate this dynamics, we have performed extensive simulations on the Gloeobacter violaceus pentameric LGIC (GLIC), a prokaryotic homologue that has been extensively used as a model since the publication of its structure in the open state two years ago. Contrary to most LGIC activated by various neutransmitters (acetylcholine, serotonin, glycine and GABA), GLIC's agonist has not been identified. GLIC is open at acidic pH, and closed at neutral and basic pH. We started from the open structure of GLIC and set it to a neutral pH, expecting a closing event. This approach appears to be successful on multi-microsecond scale, and reveals a new closed state of GLIC. This new state reveals a symmetrical organization of M2 helices, where the extracellular part of the helix is organized in a similar way as ELIC structure. Moreover, our unconstrained simulation covering four microseconds details the succession of events leading from an open to a close pore; (i) Extracellular domain (ECD) reorganization (0-0.2 μs), (ii) rotation and twist of M2 helix (0.2-0.8 μs), (iii) at 0.8 μs the pore start to dehydrate, and is followed by slow relaxation of the whole structure (0.8-4.0 μs).

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Study of the Interaction Between General Anesthetics and a Bacterial Homologue to the Human Nicotinic Receptor

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Pentameric Ligand Gated Ion Channels (pLGICs) are membrane receptors widely spread in the animal kingdom that play a key role in the nervous signal transduction. The determination of a crystal structure of one of these proteins has been recognized as particularly difficult. The discovery and the crystallization of bacterial homologues, such as GLIC from Gloeobacter violaceus in 2008 (Bocquet et al., Nature, 2008, 457:111; Hilf & Dutzler, Nature, 2008, 457:115) provided new insights in the understanding of the operating mechanism of these channels.

Recently, two general anesthetics (GAs), propofol and desflurane, have been co-crystallized with GLIC (Nury et al., Nature, 2011, 469:428). Several experimental and theoretical studies suggested the potential existence of several binding sites for GAs, alcohols and ions that modulate the activity of the channel. Such data inspire studies on the mechanism of general anesthetics and allostery within the pLGIC family.

We intend to study the dynamic properties of the interactions between GLIC-desflurane and GLIC-propofol by means of molecular dynamics simulations, on the basis of existing crystal structures. The currently available data do not allow the characterization of this interaction in a satisfying manner. We computed more than one hundred all-atom simulations of the ligand-bound GLIC system inserted in a membrane. We wish to obtain significant statistics on the exploration of the cavity by desflurane and propofol varying the initial conditions for each simulation. This approach may allow us to explore the ligand-filled cavity rather exhaustively and provide a sound bakckground for deriving hypotheses on GAs.

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Characterization of Gating Mechanisms in Prokaryotic Pentameric Ligand-Gated Ion Channels

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Pentamericligand-gated ion channels (pLGIC) mediate fast neurotransmission in the central and peripheral nervous systems and thereby regulate excitability, neurotransmitter release and muscle contraction. Upon activation, these channels rapidly switch between closed, open and desensitized conformations. The recent crystal structures of the prokaryotic pLGIC homologs from *E. chrysanthemi* (ELIC) and *G. violaceus* (GLIC) in presumably the closed and open channel conformations, respectively have provided excellent models for an atomic-level understanding of the channel structure and its role in selective ion-conduction and drug binding. However, it is still unclear as to what functional states of the pLGIC gating-scheme do these crystal structures represent. Much of this uncertainty arises from a lack of thorough understanding of the functional properties of these prokaryotic channels. Here we have carried out an extensive characterization of GLIC function by both macroscopic and single-channel current measurements in reconstituted proteoliposomes. We find that GLIC channels show rapid activation (milliseconds timescale) upon jumps to acidic pH followed by a time-dependent loss of conductance (seconds timescale) due to desensitization. We have investigated various aspects of channel activation and desensitization and determined how these gating events are modulated by pH, voltage, permeant ions, pore-blockers and membrane lipid compositions. Analyzed in the framework of recent structures, these results uncover several important features of gating mechanisms in GLIC and how they relate to their eukaryotic counterparts.

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Ionontropic Glutamate Receptors: Insight into the Mechanism of Desensitization and Deactivation using Molecular Dynamics Simulations

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Ionotropic glutamate receptors (iGluRs) mediate fast neurotransmission and are therefore essential to memory and learning processes in the brain. They are also involved in many neuropathologies, like epilepsy, Alzheimer's disease, Parkinson's disease and schizophrenia [1]. Although a large amount of structural data is currently available for iGluRs [2] and the general mechanism of functioning is well understood, the details of this mechanism at atomic level are unknown. In these conditions, molecular dynamics can be an useful tool for the study of these receptors [3].

In this work, we have designed protocols that are able to simulate in a reproducible manner, using molecular dynamics, the desensitization and the activation processes for the iGluRs Ligand Binding Domain (LBD) dimer. For the first time, we have generated a desensitized conformation of iGluRs LBD dimer without artificial structural constraints. The results obtained in this study should allow a better understanding of the factors governing these two processes and the design of new allosteric modulators for iGluRs.

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Conformational Mobility of S1-M1 and S2-M4 Linkers Influence NMDA Receptor Gating

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NMDA receptors are tetrameric glutamate-gated ion channels pivotal to excitatory neurotransmission in the mammalian central nervous system. Each subunit of the NMDA receptor harbors an extracellular ligand-binding domain (LBD) connected by three linkers to the transmembrane segments that form the ion channel. Activation gating of NMDAR involves the propagation of ligand-induced conformational changes in the LBD into ion channel pore opening via linker peptide segments. The M3-S2 linker, which is attached to the pore-lining M3 transmembrane segment, has been shown to be a critical gating element. Constraining the conformational freedom of M3-S2 relative to the adjacent S2-M4 linker of the same subunit drastically reduced the efficacy of gating with a 30-75-fold reduction in channel open probability. Here we report the gating effects of limiting the relative conformational mobility of the S1-M1 and S2-M4 linkers with crosslinking disulfide bonds. Both intra- and inter- subunit crosslinking strategies in GluN1/GluN2A-containing receptors were used to dissect the linkers' roles in gating transduction. We found that although the S1-M1 and S2-M4 linkers are connected respectively to the M1 and M4 transmembrane segment located peripheral to the pore, constraining their relative movements significantly hampered gating efficacy, as assayed using single channel recording. These results suggest that all the linkers in a single receptor complex function as an intricate unit in the gating transduction process and have important implications for potential drug targeting as unique noncompetitive antagonists are known to bind to the S1-M1 and S2-M4 linkers of certain glutamate receptor subtypes, although their mechanisms of action are yet undefined.

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An Automated Method to Study Oligomerization of Single Membrane-Bound Proteins using Fluorescence Imaging

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The stoichiometry and number of subunits comprising membrane proteins can be addressed using single-molecule fluorescence imaging. Recombinantly-expressed subunits fused genetically to a fluorescent protein are subjected to a fluorescence intensity recording. The number of fluorescently labelled subunits within a single oligomer can then be determined by counting the photobleaching steps observed as each fluorescent label loses permanently its fluorescence by photochemical destruction (*Ulbrich and Isacoff, 2007, Nature Methods*). Although this technique is powerful, drawbacks arise concerning the analysis, which is time consuming and could be biased by the user. The counting process is usually done manually, since our brain has a good ability for step detection. However, imperfections of the traces caused by background fluorescence and fluorescence blinking could be interpreted differently from one user to another. To address these issues of objectivity and productivity, we have developed a fully automated algorithm for the accurate and impartial analysis of photobleaching step counting data. After automatically selecting a spot to analyze, this analysis program extracts the step-like behaviour from a noisy trace by ''Progressive Idealization and Filtering'' (PIF). The quality of the trace and the fit are also determined in order to reject the traces which are not showing clear step-like events. Prior analysing real data with the automated routine, simulations were made to evaluate its step detection accuracy and limitations. Using PIF, we analyzed the composition of a homomeric kainate-subtype ionotropic glutamate receptor, GluK2 recombinantly expressed in HEK293T cells. GluK2 was fused with a modified version of the superfolder GFP (*Pedelacq et al., 2006, Nature Biotechnology*) and we confirmed its known tetrameric architecture in mammalian cells.

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Wavelet Shrinkage to Resolve Single Molecule FRET Structural Landscape of the Isolated Ligand Binding Domain of the AMPA Receptor

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Single molecule fluorescence resonance energy transfer (smFRET) spectroscopy has provided significant advances in our understanding of the relationship between structure and function in biological systems. Currently, simplifications must be made for experimental systems, data analysis, and theoretical modeling because biomolecules often exhibit mechanistic or conformational heterogeneity. For example, it is often necessary to treat biomolecular processes as transitions between two well-define states (e.g. folded vs. unfolded) despite clear experimental evidence or theoretical predictions to the contrary. The conformations explored by the agonist binding domain of the α-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor represent such a system. The distribution of conformations in the raw data was so wide it was not possible to extract conformational details. By employing a model-free data analysis technique called wavelet shrinkage, it was determined that each protein form comprised multi-state, sequential equilibria. The results illustrate that the extent of activation is dependent not on a rigid closed cleft, but instead on the probability that a given subunit will occupy a closed cleft conformation, which in turn is determined by the range of states that the protein explores. Also, the results emphasize both the need for and the utility of advanced data processing techniques to quantify structure and dynamics in heterogeneous systems.

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Phosphoinositide Signaling Regulates the Surface Localization of the δ2 Ionotropic Glutamate Receptor

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The δ2 glutamate receptor (GluD2) is a member of the ionotropic glutamate receptor family. Since GluD2 remains an orphan receptor, it has not been shown to conduct ions. It is highly expressed in the parallel fiber-Purkinje cell (PF-PC) synapse and its role in cerebellar physiology is increasingly appreciated. GluD2 has been linked to the induction of cerebellar long-term depression (LTD) and presynaptic terminal differentiation. A single point mutation in the second transmembrane domain (A654T), named Lurcher ($GluD2^{Lc}$), confers constitutive activity to the receptor.

We have used the Lurcher mutant as a model to examine whether phosphoinositides regulate GluD2. We show that decreasing PIP_2 levels in the membrane, through Gq-coupled receptor activation or pretreatment with a PI4K inhibitor, potentiates $GluD2^{Lc}$ currents. Conversely, increasing PIP_2 levels by co-expressing PIP5K leads to decreased $GluD2^{Lc}$ currents. On the other hand, utilizing PI3K inhibitors wortmannin or LY294002, and presumably decreasing PIP_3 levels, reduces $GluD2^{Lc}$ currents, while co-expression of PI3K leads to potentiation of $GluD2^{Lc}$ currents.

A chemiluminescence-based assay that quantifies surface localization of the GluD2 and $GluD2^{Lc}$ receptors, showed that manipulations of the membrane phosphoinositide levels evoke changes in the cell surface localization of both wild-type and mutant receptors. These changes in surface localization of the receptor correspond to the effects we have observed by monitoring $GluD2^{Lc}$ currents, suggesting that current measurements from this mutant receptor serve as a good reporter for the localization of the wild-type δ2 receptor. These results are consistent with the interpretation that increased PIP_2 levels decrease, while increased PIP_3 levels increase localization of the receptor at the cell surface. Signals that affect the levels of these phosphoinositides simultaneously are likely to regulate the surface localization of GluD2 based on the net change of the two opposing effects.

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Purification and In Vitro Functional Analysis of Glutamate Receptor

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Ionotropic glutamate receptors are predominantly localized to neuronal synapses and are principal mediators of excitatory neurotransmission in the brain. Receptor function is regulated by multiple factors including ligands, ions, pH, posttranslational modification, subunit composition, RNA editing, and elements of the lipid environment. Analysis of channel function in native membranes has provided much quantitative information on these various regulatory features, however, investigation of lipid regulation is seriously hampered by an inability to control membrane composition in living cells. Although an inhibitory effect of free polyunsaturated fatty acids on GluK2(R) activation has been demonstrated, the specific bulk lipid requirements for normal channel operation, as well as susceptibility to fatty acid modulation, are unknown. To address this, we aim to purify rat kainate receptor 2 (GluK2), to reconstitute the purified protein, and to assess channel function under different defined lipid environments. Using Saccharomyces cerevisiae as an expression host, we have been able to express wild type GluK2 with either Gln or Arg at the editing site, and with different tags. In addition, we have expressed modified forms of GluK2 including an amino terminal domain (ATD) deletion mutant, and a double point mutation (Y590C/L572C) that allows disulfide crosslinking between ligand binding domain (LBD) dimers. We have succeeded in purifying wild type GluK2(R) through Flag affinity and subsequent size exclusion chromatography after solubilizing the protein using the detergent Foscholin-14 (F14). A major band at the expected monomer size of ~100kDa and several bands at lower molecular weights are resolved on 1D SDS PAGE and are confirmed to be GluK2 through mass spectrometry (MS) analysis. MS analysis also reveals that a fraction of the heterologously expressed protein in yeast maintains a signal sequence at the N-terminus and is phosphorylated on a presumed extracellular residue, indicating misoriented topology.

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Activation of Ionotropic Glutamate Receptors using Tethered Photoswitchable Ligands

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Ionotropic glutamate receptors (iGluRs) are ligand-gated ion channels, which mediate and modulate excitatory neurotransmission in the central nervous system. iGluRs are tetramers that are formed by identical or homologous subunits, but little is known about how ligand binding at individual subunits contributes to gating. Here we address this question using photoswitchable ligands that are tethered to specifically introduced cysteine residues. The ligand, termed MAG, consists of a cysteine-reactive maleimide group, an azobenzene photoswitch, and a glutamate analogue as head group. After coupling to an attachment site close to the binding cleft, light of specific wavelengths can be used to switch MAG between its cis and trans form, which allows to control ligand binding and unbinding with high temporal precision. We combined photoswitching with voltage-clamp recordings to measure the effect of partial receptor occupation on the extent and time course of activation and desensitization in GluK2, a member of the kainate receptor family. Extending these experiments to heteromeric complexes and complexes of defined subunit stochiometry will provide further information on how individual subunits contribute to the activation, deactivation and desensitization of this important class of signaling molecules.

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N-Terminal Domain of NMDA Receptors as Studied by LRET

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NMDA Receptors, one of the three main classes of glutamate receptors that mediate excitatory transmission, are tetramers composed of glycine- binding (GluN1) subunits and glutamate- binding (GluN2) subunits. Allosteric